RESEARCH ARTICLE
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Multigene Panel Testing Revealed Novel Variants in Hereditary Spherocytosis Patients in Türkiye

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Abstract:
Objective: This study aims to determine the genotypic characteristics of Hereditary Spherocytosis (HS) patients in Turkiye and to examine the correlation between genotype and phenotype.
Materials and Methods: Herein we had 18 patients who were admitted to pediatric hematology outpatient clinic with hemolytic anemia, jaundice, cholelithiasis, and splenomegaly. According to the Eber's classification, the patients' clinical presentations were categorized as mild, moderate, and severe. The next-generation sequencing method was used to analyze single nucleotide and copy number variations in all genes associated with HS via clinical exome sequencing (CES). The relationship between the genes with detected variants and the clinical presentation in the patients was investigated.
Results: In total, 21 variants were detected in five HS-related genes. Twelve of them were previously reported variants, and nine of them were novel variants. Seven of them were pathogenic and two of them were classified as Variant of Uncertain Significance (VUS) according to the American College of Medical Genetics and Genomics (ACMG). Herein we have discussed the phenotypic effects of novel pathogenic variants in *SPTA1*, *SPTB*, *ANK1*, *SLC4A1*, and *EPB42* genes. Patients with *EPB42* and *SLC4A1* gene pathogenic variants had less severe clinical findings compared to other gene variants according to Eber's classification. On the other hand, patients carrying pathogenic variants of *SPTA1* and *SPTB* genes had more severe clinical presentation.

Conclusion: Molecular diagnosis of HS is important for treatment, prediction of the clinical outcome, and appropriate genetic counseling. As a result, our study contributes to the genotype-phenotype distribution of HS by introducing novel variants to the literature.
Keywords: Hereditary Spherocytosis, HS, NGS, Genotype-Phenotype Correlation

Özet:
Amaç: Bu çalışmanın amacı Türkiye'deki herediter sferositoz hastalarının genotipik özelliklerini belirlemek ve genotip ile fenotip korelasyonunu incelemektir.
Gereç ve Yöntemler: Bu çalışmada, hemolitik anemi, sarılık, safra taşı ve splenomegali ile çocuk hematoloji polikliniğine başvuran 18 hasta incelenmiştir. Eber sınıflandırmasına göre hastaların klinik bulguları hafif, orta ve ağır olarak kategorize edilmiştir. Herediter Sferositoz (HS) ile ilişkili tüm genlerdeki tek nükleotid ve kopya sayısı değişikliklerini analiz etmek için klinik ekzom dizileme kiti ile yeni nesil dizileme yöntemi kullanılmıştır. Hastalarda varyant saptanan genler ile klinik prezentasyon arasında ilişki olup olmadığı araştırılmıştır.
Bulgular: HS ilişkili beş gende toplam 21 varyant tespit edilmiştir. Bunlardan 12'si tanımlı varyantlar, dokuzu ise yeni varyantlardır. Yedi tanesi patojenik olup ikisi Amerikan Klinik Genetik ve Genomik Koleji'ne (ACMG) göre klinik önemi bilinmeyen varyant olarak sınıflandırılmıştır. Bu çalışmada, *SPTB, ANK1, SLC4A1, SPTA1* ve *EPB42* genlerindeki yeni patojenik varyantların fenotipik etkileri tartışılmıştır. Eber sınıflamasına göre, *EPB42* ve *SLC4A1* genlerinde patojenik varyantları olan hastalar diğer gen varyantlarına kıyasla daha hafif klinik bulgular göstermiştir. Öte yandan, *SPTA1* ve *SPTB* genlerinin patojenik varyantlarını taşıyan hastalar daha ciddi klinik seyre sahip olmuştur.
Sonuç: HS'nin moleküler tanısı, tedavi, klinik sonucun öngörülmesi ve uygun genetik danışmanlık için önemlidir. Sonuç olarak, çalışmamız literatüre yeni varyantlar kazandırarak HS'nin genotip-fenotip dağılımına katkı sağlayacaktır.

Introduction:

Hereditary anemias (HA) encompass a highly diverse group. Pathogenic variants in more than 70 genes cause HA (1). The most common cause of hereditary hemolytic anemia is hereditary spherocytosis (HS) (1). HS is a disease that exhibits heterogeneity both clinically and genetically. Common clinical manifestations are hemolytic anemia, splenomegaly, jaundice, and cholelithiasis. Wide clinical spectrum of HS ranges from very mild disease to very severe requiring splenectomy and transfusions. Although studies on genotype-phenotype correlation in HS have been conducted over the past ten years, a clear correlation has yet to be established (2).
As a result of pathogenic variants in genes encoding membrane or cytoskeleton proteins, spherical-shaped red blood cells (RBC) are formed (3). To date, five genes [*ANK1* (ankyrin) (OMIM:612641), *SPTA1* (a-spectrin) (OMIM:182860), *SPTB* (b-spectrin) (OMIM:182870), *EPB42* (protein 4.2) (OMIM:177070), and *SLC4A1* (band-3) (OMIM:109270)] that cause HS have been identified (4-8). *SPTA1* and *EPB42* genes are related to autosomal recessive (AR) inheritance, *SPTB,* and *SLC4A1* genes cause autosomal dominant (AD) inheritance and the *ANK1* gene is responsible for both AR and AD inheritance. Overall, AD and AR inheritance patterns are present in 75% and 25% of patients, respectively. Pathogenic variants are most commonly detected in the *ANK1* gene (40-65%) in patients with HS, followed by *SLC4A1* (20-35%) and *SPTB* genes (15-30%), respectively (9).
Due to nonspecific and overlapping clinical findings, differential diagnosis and classification of HA's are not easy. Genetic testing may be necessary for these patients to confirm the clinical diagnosis, in order to provide appropriate genetic counseling to the family, establish a genotype-phenotype correlation based on the accumulated literature, and to allow for an accurate assessment of the clinical severity and proper monitoring of the patients.
This study aims to determine the genotypic characteristics of HS patients in **our cases with CES testing** and to examine the correlation between phenotype and genotype.

Materials and Methods:

Patients

From May 2020 to September 2023, besides the presence of spherocytes on peripheral smear, patients who had a clinical diagnosis of HS and were admitted with non-immune hemolytic anemia and had negative tests for hemoglobinopathies, pyruvate kinase, and glucose-6-phosphate dehydrogenase deficiency; were analyzed with next-generation sequencing (NGS) multigene panel. The osmotic fragility test was performed on all patients, and an increase in osmotic fragility was observed in all cases. Our cohort covered 18 unrelated probands in Turkiye with HS-associated pathogenic variants. The epidemiological, clinical, and laboratory results were evaluated retrospectively. The classification of severity of disease was based on hemoglobin (Hb), reticulocyte percentage, and serum total bilirubin levels as in Eber's classification (2, 3). Based on Eber's classification the clinical presentation of HS was classified into mild, moderate, and severe forms. In the mild form, hemoglobin levels range from 11-15 g/dL, reticulocyte counts are between 3-6%, and bilirubin levels are 17-34 µmol/L, with splenectomy typically not required. The moderate form is characterized by hemoglobin levels of 8-12 g/dL, reticulocyte counts exceeding 6%, and bilirubin levels above 34 µmol/L, where splenectomy is often necessary before puberty. In severe HS, hemoglobin levels fall between 6-8 g/dL, reticulocyte counts exceed 10%, and bilirubin levels are above 51 µmol/L, with splenectomy being almost universally indicated. According to the severity of hemolysis, 7 patients were in the mild group, 6 were in the moderate group and 5 were in the severe group. Among those 5 patients in the severe group, two of them had undergone splenectomy. The local ethics committee of Marmara University School of Medicine approved the study (Approval number: 1770).

Molecular studies:

Patients' peripheral blood DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, MD, USA). Quantification of extracted DNA was performed using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). DNA with absorption ratios between 1.8–2.0 for the 260/280 nm ratio and 1.6–2.4 for the 260/230 nm ratio, and with a concentration ranging from 50-100 ng/µl, was used. Briefly, one microgram of genomic DNA was fragmented using ultrasonication (Covaris, Woburn, MA, USA). The resulting fragments were end-repaired, adapter-ligated, and subjected to size selection before being enriched. Sequencing was performed via the Illumina Nextseq 550 platform (San Diego, CA, USA). Approximately 10 GB of sequence data was generated for this sample by loading it onto a flow cell lane, producing 2×100 bp paired-end reads. The reads were mapped to the human genome reference build GRCh37/hg19 using the Burrows-Wheeler Aligner (BWA) algorithm. PCR duplicates were marked with GATK v1.64, and the output was converted to BAM format. The mean exome coverage was 66×, with 96.5% of target bases covered more than 10× and 83.8% covered more than 30×. The Sophia DDM-V4 (Boston, USA) platform was used for data analysis.

Single nucleotide variants (SNVs) and copy number variants (CNVs) of *SPTB* (NM_000347), *SPTA1* (NM_003126), *ANK1* (NM_000037), *EPB42* (NM_000119), and *SLC4A1* (NM_000342) genes were evaluated. The Clinical Exome Solution (CES) v3 (Boston, USA) panel by Sophia Genetics targets 5,800 genes associated with inherited diseases. Among these genes are those associated with congenital dyserythropoietic anemias, Diamond-Blackfan anemia, hereditary stomatocytosis, glucose-6-phosphate dehydrogenase deficiency, and pyruvate kinase deficiency. These genes were analyzed in each patient in our cohort, and patients with variants in these genes were not included in the study. Rare variants were searched in ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) and Human Gene Mutation Database (HGMD-http://www.hgmd.cf.ac.uk) to figure out whether they had been reported before. Also, variants were searched in the current literature, and if they had been previously described in the literature they were not considered novel. Pathogenicity of retained novel variants was determined following the ACMG criteria (10). Segregation was analyzed using the Illumina Miseq Platform (San Diego, CA, USA).

Results:

The laboratory and clinical findings of 18 patients in Turkiye who were found to have variants related to HS were presented in Table 1. Eleven of the patients were males (61%) and the median age was 8 years; ranging from two to 58 years. Only 6 patients had elevated mean corpuscular hemoglobin concentration (MCHC) levels.

Nine patients had *SPTB*, three patients had *SPTA1*, three had *ANK1*, two had *SLC4A1,* and two had *EPB42* variants. We have detected 21 variants in 18 patients because a patient had both *SPTA1* and *SPTB* variants (Patient 14). Out of the patients with *SPTB* variants, two cases were evaluated as mild, three as moderate, and three as severe HS.

Among two patients who had *SPTA1* variants (Patients 13, and 18) one had severe and the other had moderate HS. Three patients had *ANK1* variants (patients 5, 10, and 15, respectively), one of them had moderate and the other two had severe HS. Among the two patients with *SLC4A1* variants (Patients 1 and 8), one was classified as mild and the other as moderate HS. The two patients with *EPB42* variants (Patients 6 and 17) displayed mild clinical findings. The patient who had both *SPTA1* and *SPTB* variants (Patient 14) was classified as mild HS.

In total, 21 variants were detected in the five HS-related genes. Twelve of them were previously reported variants, and nine of them were novel variants (Table 2, Figure 1). Out of the detected variants, 10 of them caused premature termination in the protein, while 11 of them were non-truncating variants (Figure 2). A total of 9 variants were identified in the *SPTB* gene, and this gene had the highest frequency of detected variants. The number of the detected variants in the *SPTA1*, *SLC4A1*, *ANK1,* and *EPB42* genes were 3, 2, 3, and 2, respectively.

In our cohort, 9 novel variants were detected. By the ACMG criteria, seven of them were pathogenic and two of them were VUS. Four novel pathogenic variants were identified in the *SPTB* gene, two in the *SLC4A1* gene, two in *SPTA1* gene, and one in the *ANK1* gene. Of the novel variants in the *SPTB* gene, two were nonsense variants (c.5587C>T, p.Gln1863*; c.3450G>A, p.Trp1150*) (Patient 3 and 12), one was a splice-site variant (c.3561+1G>C)(Patient 9), and the other was a frameshift variant (c.4832dupA, p. Ile1612Aspfs*5)(Patient 2). The novel variant in the *ANK1* gene was a frameshift variant (c.1771dupC, p.Arg591Profs*30) (Patient 15). One of the two novel variants identified in the *SLC4A1* gene was a missense variant (c.2422C>A, p.Arg808Ser) and the other was a splice-site variant (c.1801-2A>C) (Patient 8 and 1). The novel pathogenic variant identified in the *SPTA1* gene in this study was the sole gross deletion detected, which encompassed exon 2-52 (Patient 13). Three of the novel VUS were missense variants and one was a synonym variant. Two of them (c.6026G>A, p.Arg2009His; c.3208C>A p.Arg1070Arg) were identified in the *SPTA1* gene, one (c.1195G>A, p.Ala399Thr) was found in the *ANK1* gene, and one (c.1370G>A, p.Arg457His) was found in the *EPB42* gene (Patients 14,13,5,6, respectively). All detected variants are shown in Figure 3.

Novel pathogenic variants and phenotypic effects:

The c.3450G>A variant causes a transition from guanine to adenine at position 3450 of exon 15 in the *SPTB* gene (Patient 12). This change leads to the 1150th codon becoming a stop codon, causing premature termination of the protein. This variant, which was not reported in ClinVar, has been assessed as ‘likely pathogenic’ (LP) (PVS1: Null variant in a gene where loss of function is a known mechanism of disease, PM2: Extremely low frequency in gnomAD population databases) according to the ACMG criteria. The patient was a 15-year-old girl who had mild disease, slightly increased reticulocyte percentage with normal hemoglobin levels, and no splenomegaly or hyperbilirubinemia

The c.3561+1G>C variant is located at the splice donor site of exon 15 in the *SPTB* gene (Patient 9). A change from guanine to cytosine, which this variant causes, is in the consensus donor site sequence that is typical "GT". This change is accepted to affect gene splicing, leading to abnormal protein production. According to ACMG criteria, this variant was evaluated as LP (PVS1, PM2). The patient with this variant was a 33-year-old female with moderate HS.

The duplication of adenine at position 4832 in the *SPTB* gene results in the substitution of aspartic acid, instead of isoleucine which is the 1612th amino acid and causes premature termination of the protein after the addition of 5 amino acids (Patient 2). This variant was not reported in the ClinVar database. As ACMG criteria point out; the classification of this variant is LP (PVS1, PM2). The patient was an eight-year-old boy who had splenectomized due to transfusion-dependent severe anemia.

The duplication of cytosine at the 1771th position of *ANK1* gene causes substitution of 591st amino acid arginine to proline and leads to termination after addition of 30 amino acids. This variant was also evaluated as LP (PVS1, PM2) at ACMG classification (Patient 15). The patient was a 4-year-old boy with severe HS, who had profound splenomegaly and cholelithiasis, needed frequent transfusions.

The substitution of cytosine with adenine at position 2422 of the *SLC4A1* gene results in a serine instead of arginine, which is the 808th amino acid. This missense variant is located at the 18th exon of the *SLC4A1* gene (Patient 8). Although this variant was not reported in ClinVar, an alteration that causes different amino acid replacements at the same position (p.Arg808His) had been reported as P/LP in the same database. According to ACMG criteria, the pathogenicity of this variant is evaluated as LP (PP3:

Computational prediction tools unanimously support a deleterious effect on the gene, PM2, PM5: Different amino acid change as a known pathogenic variant). The patient was a 7-year-old girl with moderate HS. She had splenomegaly and cholelithiasis, but transfusion was not required.
The c.1801-2A>C variant causes adenine to cytosine conversion in the splice-acceptor site of the *SLC4A1* gene (Patient 1). This change, with its effect on splicing, possibly leads to an abnormal protein product. The heterozygous c.1801-2A>C variant in the *SLC4A1* was not reported in ClinVar; however, a change from A to G at the same position was described in ClinVar as LP. This supports the pathogenicity of the variant identified in our patient (PM5). The variant has been assessed as LP at the ACMG classification (PVS1, PM2, PM5). The patient was a 58-year-old male with mild HS.
Deletion of the entire *SPTA1* gene was reported previously (11); however, deletion of exon 2-52 of *SPTA1* gene has not been reported (Patient 13). This deletion ends up with a truncated protein (PVS1). Additionally, this variant was rare in population databases (PM2). According to ACMG criteria, the pathogenicity of this variant was evaluated as LP. The patient was an 8-year-old boy, who had moderate HS, splenomegaly, cholelithiasis, and necessitating intermittent erythrocyte transfusions.

Discussion:
Hereditary Spherocytosis is the most frequent form of non-immune hemolytic anemia, exhibiting a prevalence ranging from 1/2000-5000 (1,3,10). While most variants manifest through dominant inheritance, AR transmission is also evident in cases involving *SPTA1* and *EPB42* variants (1,7,9). The genetic landscape of HS is influenced by ethnicity and race, contributing to diverse variations within populations. Numerous studies conducted in Europe and North America (2,9,11,12) have identified *ANK1* and *SPTB* variations as primary contributors to genetically defined HS cases, whereas in Japan (7), *EPB42* and *SLC4A1* variants were recognized as the most prevalent aberrations. However, comprehensive data on the prevalence of these variations remains limited in Turkiye.
In this study, 18 patients had 21 variants detected in the genes *SPTB, SPTA1, EPB42, SLC4A1,* and *ANK1* with 9, 5, 2, 2, and 3 variants, respectively. Notably, one patient in our series presented with combined pathogenic variations in *SPTA1* and *SPTB* genes. Spectrin variants emerged as the most frequently observed genetic alterations, accounting for 11 out of 18 cases (61%) in our cohort.
Although high MCHC is typically considered a useful parameter, studies show that it has a weak parameter for diagnosis of HS (13). Similarly in our series, we observed elevated MCHC levels in only 6 out of 18 patients (33%).

Genotype-phenotype correlation
Hereditary Spherocytosis displays significant heterogeneity. Studies focused on genotype-phenotype correlation in clinical settings are still a subject of controversy. Although the most common pathogenic variants were found in *ANK1* gene in studies conducted in Northern Europe and the USA, the most common variant was found in *SPTB* gene in our study (9). Two cases had mild, 3 had moderate, and 3 had severe HS. A patient had combined variants of *SPTA1* and *SPTB*. In our series we had two cases with *SPTA1* variants, one had moderate and the other had severe HS and had splenectomy. Similarly, in a study conducted in Canada, it was observed that children with pathogenic variants in the *SPTA1* gene had the lowest hemoglobin values and were more likely to require transfusions and undergo splenectomy and cholecystectomy in childhood than patients with all other forms of HS (14). *SLC4A1* variants are generally reported to cause mild compensated hemolytic anemia and are often diagnosed in adulthood. *SLC4A1* variants were detected in two of our patients, the patient, who had mild HS, was 58 years old whereas the other with moderate HS was eight years old. Two extensive sample studies conducted in Canada and Netherlands revealed that pediatric patients exhibiting *SLC4A1* defects manifested a less severe phenotype (9, 14). In another study, children with HS with a pathogenic variant in the *SLC4A1* gene had the mildest phenotype (9). They had the highest hemoglobin, lowest reticulocyte count, and lowest unconjugated bilirubin levels compared to patients with pathogenic variants in other genes. In addition, none of them required splenectomy in childhood. Although *EPB42* has been reported to be very rare and, usually frequent in Japanese, two (11.1%) of 18 patients had EPB42 variants (7). Both cases had mild HS, relevant to the patients reported in the literature (7, 9). Three patients had *ANK1* variants, two had severe, and the other had moderate HS (patients 5, 10, and 15). Van Vuren et al. reported that pathogenic variants

in the spectrin binding domains of *ANK1*, *SPTA1,* and *SPTB* cause a more severe phenotype (9). Although our study found that patients with *ANK1* variants had more severe hemolytic findings, a larger sample size is needed.

As a limitation of our study, functional studies such as animal experiments or cell cultures to evaluate the detected VUS variants could not be performed. Therefore, further studies are necessary. In addition, this study is a single center study and more patients are needed to make clear assessments of the genotype-phenotype correlation in HS patients.

In conclusion, this is the first cohort study in Turkiye on genotype-phenotype correlation in patients with HS. In addition, nine novel variants were introduced to the literature. In our cohort, *SPTB* was the gene that had the most frequent variants and presented with a wide clinical spectrum. The patients with *ANK1* and *SPTA1* variants showed more severe clinical features, while patients with *EPB42* and *SLC4A1* variants were observed to have mild clinical findings. In the differential diagnosis of non-immune hemolytic anemias, nonspecific parameters such as high MCHC are being replaced by NGS analysis.

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Conflict of interest statement: None

Abbreviations:
ACMG: American College of Medical Genetics and Genomics
AD: Autosomal Dominant
AR: Autosomal Recessive
CNV: Copy number variant
Hb: Hemoglobin
HGMD: Human Gene Mutation Database
HS: Hereditary Spherocytosis
MCHC: Mean Corpuscular Hemoglobin Concentration
NGS: Next-Generation Sequencing
RBC: Red Blood Cells
Ret: Reticulocyte
SNV: Single nucleotide variant
VUS: Variant of Uncertain Significance

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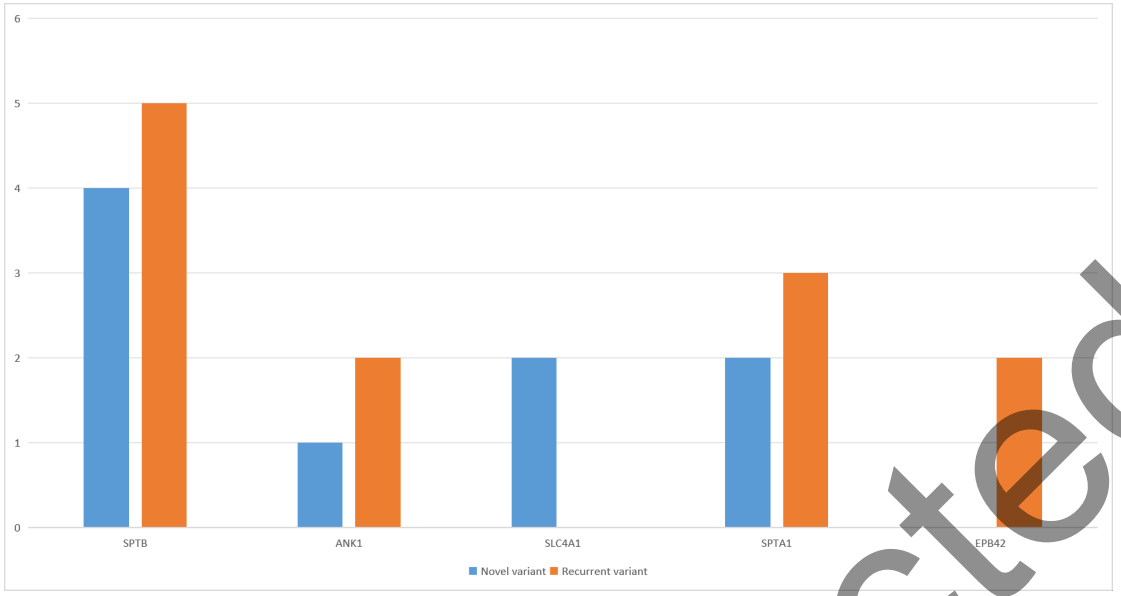

Figure 1. Presentation of previously reported and novel variants in five HS-related genes.

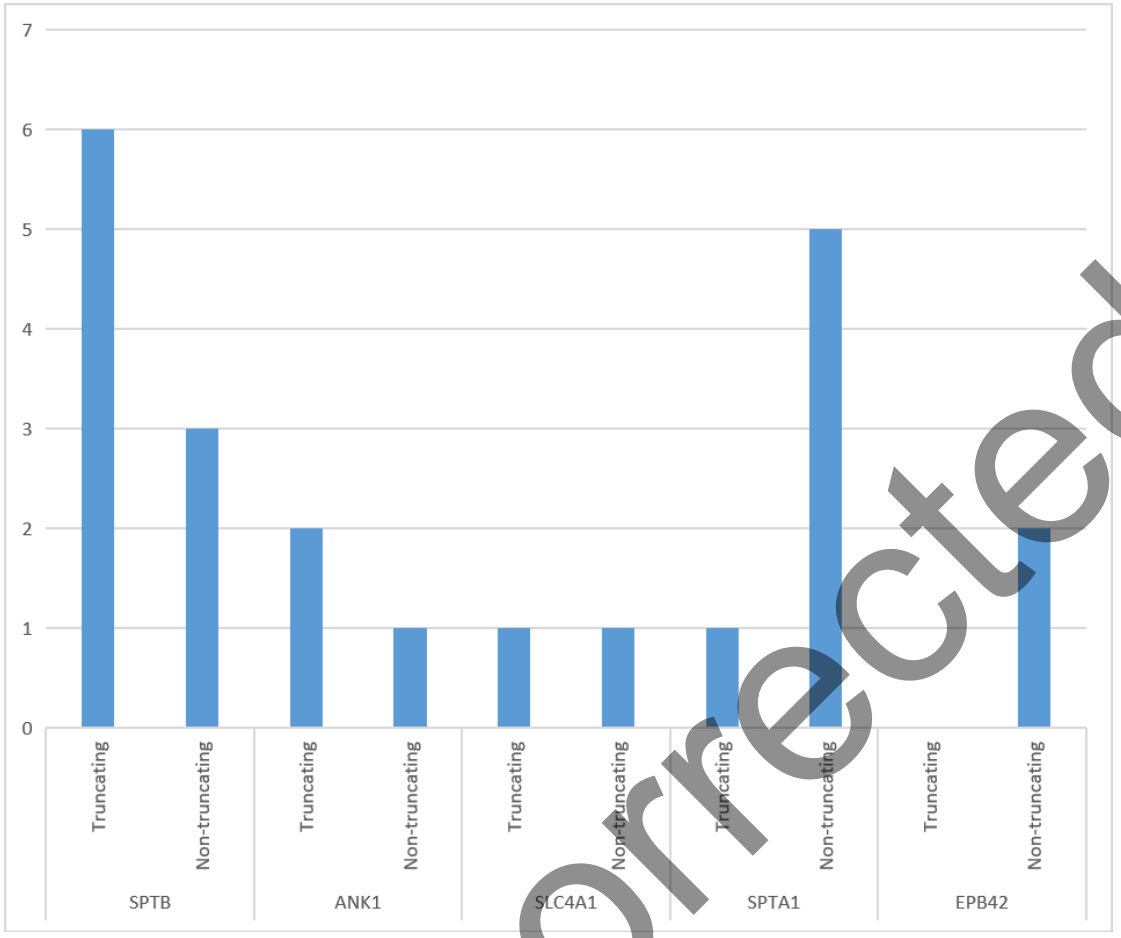

Figure 2. Representation of truncating and non-truncating variants identified in the HS-related genes

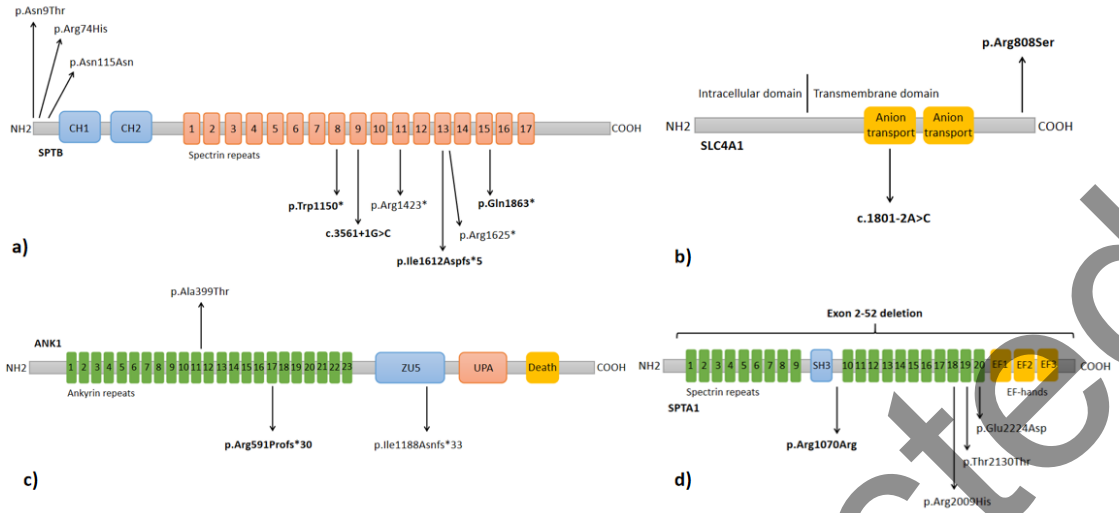

Figure 3. Schematic presentation of all variants identified in the study on the protein domains of a) *SPTB*, b) *SLC4A1*, c) *ANK1*, and d) *SPTA1*. Novel variants are shown in bold.

Table 1. Clinical and Laboratory Findings of Patients with HS.

ID	Sex	Age	Severity	Hb	Ret	Bilirubin	MCHC	USG Splenomegaly	Cholelithiasis	Transfusion	Splenectomy
Patient 1	M	58	mild	12,7	7,2	2,0	37,3	No	No	No	No
Patient 2	M	8	severe	6,9	5,1	4,6	34,1	Yes	Yes	Yes	Yes
Patient 3	M	4	moderate	8,2	11,0	3,3	33,6	Yes	Yes	No	No
Patient 4	M	8	severe	6,7	10,8	7,8	34,2	Yes	Yes	Yes	Yes
Patient 5	M	3	moderate	9,9	6,0	0,2	34,0	Yes	Yes	No	No
Patient 6	F	5	mild	11,7	4,4	3,2	34,7	No	No	No	No
Patient 7	F	9	moderate	8,8	14,5	3,4	34,1	Yes	Yes	Yes	No
Patient 8	F	7	moderate	9,9	9,9	3,4	36,1	Yes	Yes	No	No
Patient 9	F	33	moderate	10,1	8,6	2,8	34,9	No	No	No	No
Patient 10	M	3	severe	6,8	9,8	3,0	31,9	Yes	Yes	Yes	No
Patient 11	M	10	mild	12,9	0,9	2,3	33,9	Yes	Yes	No	No
Patient 12	F	15	mild	13,3	3,2	0,8	37,0	No	No	No	No
Patient 13	M	8	moderate	8,9	6,7	6,9	32,4	Yes	Yes	Yes	No
Patient 14	M	19	mild	13,2	1,2	2,1	35,7	Yes	Yes	No	No
Patient 15	M	4	severe	7,0	16,3	1,9	35,5	Yes	Yes	Yes	No
Patient 16	M	44	mild	13,5	3,4	0,6	33,2	Yes	NA	No	No

Patient 17	F	2	mild	11,3	4,4	1,0	34,5	Yes	Yes	No	No
Patient 18	F	6	severe	7,1	4,6	1,7	39,0	Yes	Yes	Yes	No

Hb, hemoglobin; Ret, reticulocyte; MCHC, mean corpuscular hemoglobin concentration. Hb (g/dL) ref: 11.5-15.0; Ret % ref: 0.5-2; MCHC (g/dL) ref: 31.6–35.4. The Hb levels before splenectomy of patients.

Table 2. Detailed bioinformatic results of the variants identified in 18 patients in Turkiye diagnosed with HS

Patient	Gene	cDNA	Protein	Zygosity	Variant Type	Clinvar	ACMG	Reference
Patient 1	*SLC4A1* (NM_000342)	c.1801-2A>C	-	Heterozygous	Splice-site	Not reported	LP	This Study
Patient 2	*SPTB* (NM_000347)	c.4832dupA	p.Ile1612Aspfs*5	Heterozygous	Frameshift	Not reported	LP	This Study
Patient 3	*SPTB* (NM_000347)	c.5587C>T	p.Gln1863*	Heterozygous	Nonsense	Not reported	LP	This Study
Patient 4	*SPTB* (NM_000347)	c.345T>C	p.Asn115Asn	Heterozygous	Synonymous	VUS/Benign	LB	-
Patient 5	*ANK1* (NM_000037)	c.1195G>A	p.Ala399Thr	Heterozygous	Missense	VUS	VUS	-
Patient 6	*EPB42* (NM_000119)	c.1370G>A	p.Arg457His	Heterozygous	Missense	VUS	VUS	-
Patient 7	*SPTB* (NM_000347)	c.4873C>T	p.Arg1625*	Heterozygous	Nonsense	Pathogenic	P	Agarwal et al (2016)
Patient 8	*SLC4A1* (NM_000342)	c.2422C>A	p.Arg808Ser	Heterozygous	Missense	Not reported	LP	This Study
Patient 9	*SPTB* (NM_001024858)	c.3561+1G>C	-	Heterozygous	Splice-site	Not reported	LP	This Study
Patient 10	ANK1 (NM_000037)	c.3563_3564del	p.Ile1188Asnfs*33	Heterozygous	Frameshift	Likely Pathogenic	LP	-
Patient 11	*SPTB* (NM_000347)	c.4267C>T	p.Arg1423*	Heterozygous	Nonsense	Pathogenic	P	Peng GX et al (2018)
Patient 12	*SPTB* (NM_001024858)	c.3450G>A	p.Trp1150*	Heterozygous	Nonsense	Not reported	LP	This Study
Patient 13	*SPTA1* (NM_003126)	c.3208C>A Exon 2-52	p.Arg1070Arg -	Heterozygous Heterozygous	Synonymous Deletion	Not reported Not reported	VUS -	This Study This Study
Patient 14	*SPTB* (NM_000347) *SPTA1* (NM_003126)	c.26A>C c.6026G>A	p.Asn9Thr p.Arg2009His	Heterozygous Heterozygous	Missense Missense	VUS VUS	VUS VUS	Russo R et al (2018)
Patient 15	*ANK1* (NM_000037)	c.1771dupC	p.Arg591Profs*30	Heterozygous	Frameshift	Not reported	LP	This Study
Patient 16	*SPTB* (NM_000347)	c.221G>A	p.Arg74His	Heterozygous	Missense	VUS	VUS	-
Patient 17	*EPB42* (NM_000119)	c.1477G>A	p.Gly493Ser	Heterozygous	Missense	VUS	B	-

Patient 18	*SPTA1* (NM_003126)	c.6672A>C c.6390C>T	p.Glu2224Asp p.Thr2130Thr	Heterozygous Heterozygous	Missense Synonymous	LP/VUS/LB/B VUS	LB VUS	- -

P: Pathogenic, LP: Likely Pathogenic, VUS: Variant of Unknown Significance, LB: Likely Benign, B: Benign